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Therapeutic Implications of Progesterone Receptor-Mediated Regulation of Cell Cycle in Breast Cancer

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14. ABSTRACT

Since the 2007 summary report, we have made significant progress in elucidating the novel mechanisms by which PR regulates expression of E2F1, a key regulator of cell cycle progression, in T47D breast cancer cells. In addition to a direct regulatory pathway involving recruitment of PR to the E2F1 promoter, we have identified several indirect modes of regulation. First, ligand-bound PR stimulates increased recruitment of E2F1 to its own promoter in a positive feedback loop. Second, treatment with the synthetic progestin R5020 induces expression of KLF15, a member of the Sp/KLF superfamily of transcription factors, which can then bind to GC-rich DNA within the E2F1 promoter and further amplify transcriptional activation. Finally, we have discovered that lower concentrations of the synthetic progestin R5020 are better able to mediate induction of E2F1 than higher concentrations of R5020. This means that the current paradigm of giving low dose progestins in combination with estrogen in HRT and contraceptives may actually be increasing the activation of certain downstream PR target genes, including those involved in cell cycle progression and proliferation.

15. SUBJECT TERMS
Progesterone receptor, E2F, cell cycle, KLF15

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Introduction

Progesterone is a naturally occurring steroid hormone that functions by binding to the progesterone receptor (PR) and thereby enabling the receptor to bind DNA, recruit cofactors, and induce the transcription of target genes [1]. In addition, PR indirectly regulates gene expression through a rapid, non-genomic pathway by interacting with Src family kinases and signaling through downstream MAPK [2]. In the breast and other tissues of the female reproductive system, progesterone plays an key role in normal development and function [3]. However, recent data suggests that PR also contributes to the proliferation of breast cancer cells [4, 5]. Given that over half of all breast cancers express PR, antiprogestins which inhibit the proliferative functions of PR may have the potential to block breast cancer progression [6]. Therefore, the goal of this project is to explore the mechanisms by which PR regulates breast cancer proliferation so that we can better enable development of PR modulators (PRMs) that effectively inhibit breast tumor growth. Our studies focus on a novel pathway by which PR may control proliferation of T47D breast cancer cells. In a preliminary microarray study done on T47D breast cancer cells, we found that pre-treatment with the MEK 1/2 inhibitor U0126 altered PR-mediated regulation of a group of over 1000 genes. We discovered that the promoters of a subset of these PR-regulated genes were enriched for E2F binding sites. Since E2F family members are key transcription factors required for regulation of cell cycle, we decided to investigate PR regulation of E2F signaling.

Body

In most breast cancer cell lines, estrogens are important for regulation of PR expression; however, estrogen is a known mitogen in breast cancer, and has previously been shown to induce expression of E2F1 [7]. Since we wanted to concentrate solely on PR-specific regulation of E2F expression and cell cycle progression, we chose the T47D cell line as a model system for our studies because PR functions are uncoupled from ER signaling in this breast cancer cell line.

Previous Data

The **first task** for this project was to define the conditions under which PR positively regulates E2F transcription. In previous studies reported in the 2007 Annual Summary, we used real-time qPCR and Western blot analysis to determine that treatment of T47D cells with R5020 induces expression of E2F1 at both the mRNA and protein levels (**Figures 1 & 13**). We observed that pre-treatment with the MEK 1/2 inhibitor U0126 blocked R5020-mediated induction of E2F1, indicating that the rapid, non-genomic actions of PR may be partly responsible for its regulation of E2F1. In addition, treatment with R5020 induces transcription of downstream E2F1 target genes such as cdc2 and cdc6.

To ensure that PR is necessary for R5020-mediated induction of E2F transcription, we obtained T47DC42 cells [PR(-) T47D subclone] that stably express a LacZ control, wild-type PR-A, or wild-type PR-B from Dr. Dean Edwards. Real-time qPCR analysis shows that R5020 induces E2F1 mRNA in cells expressing PR-B alone (**Figure 4B**), but not in cells expressing the LacZ control or PR-A alone (**Figure 2)**. Next, we established that regulation of E2F1 signaling by PR-B is not a unique phenomenon that is restricted to T47D cells. R5020 induces expression of E2F1 in BT483 breast cancer cells (**Figure 3A**), which have normal coupling of the ER and PR pathways, and in ER-/PR- human mammary epithelial cells (HMECs) infected with a PR-B adenovirus (**Figure 3B**).

Our **second task** was to assess the relative contributions of genomic vs. non-genomic PR actions on E2F signaling. Given that R5020-mediated induction of E2F1 is blocked by U0126, we initially thought that the rapid, non-genomic actions of PR may be partly

responsible for its regulation of E2F. To further investigate the potential contribution of non-genomic PR signaling to E2F activity, we obtained a T47DC42 PRBmPro cell line from Dr. Dean Edwards that stably expresses a mutant form of PR-B in which three key proline residues in the polyproline motif were replaced with alanines. This mutant PR receptor is unable to mediate rapid, non-genomic activation of Src family kinases or downstream MAPK, but its classical genomic functions remain intact. Contrary to our initial hypothesis, we found that R5020 induces equal expression of E2F1 mRNA in cells expressing wild-type PR-B versus the mutant PRBmPro version (**Figure 4B**). Furthermore, pre-treatment with the Src family kinase inhibitor SU6656 does not abrogate PR-mediated induction of E2F1 mRNA (**Figure 4A**). These data seem to indicate that although the MAPK pathway is important for phosphorylation of RB and release of E2F, its activation is not dependent on PR signaling through Src family kinases.

To address whether PR directly regulates the transcription of E2F by binding to its promoter, we performed chromatin immunoprecipitation (ChIP) experiments using synchronized T47D cells treated with vehicle or R5020. ChIP analysis shows that agonist-bound PR is recruited to the E2F1 promoter, suggesting that PR functions to directly induce E2F1 transcription (**Figure 5**). However, at the time of the 2007 Annual Summary, we had been unable to identify the exact DNA region to which PR binds because we could not locate any classic progesterone response elements (PREs) within the E2F1 promoter.

Current Data

During the present reporting period, we had planned to further define the direct regulation of E2F1 by PR by carrying out more detailed motif searches to identify non-canonical or half-PRE sites within the E2F1 promoter. However, in a recent collaboration with the laboratory of Myles Brown at the Dana-Farber Cancer Institute at Harvard Medical Center, we have received preliminary ChIP-chip data which reveals that PR binds to two DNA sites proximal to E2F1. We intend to conduct additional ChIP studies to verify that PR is recruited to these two regions of DNA.

However, pre-treatment of T47D cells with cycloheximide, which blocks *de novo* protein synthesis, partially inhibits R5020-mediated induction of E2F1 (**Figure 6**). This data indicates that direct recruitment of PR to the E2F1 promoter is not the only pathway by which PR regulates E2F1. In additional ChIP experiments conducted during the present reporting period, we discovered another indirect pathway by which PR induces E2F1 expression. Treatment of T47D cells with R5020 for as little as 60 minutes significantly increases recruitment of E2F1 to its own promoter, forming a positive feedback loop that further amplifies its transcription (**Figure 5**).

In order to determine what other factors could be involved in PR induction of E2F1 transcription, we decided to take a closer look at the E2F1 promoter. To accomplish this, we obtained a series of reporter gene constructs from Dr. Joseph Nevins that contain successively smaller regions of the E2F1 promoter, and also some point mutants that lack E2F binding sites A, B or both (**Figure 7A**). Importantly, the 82-bp region that was removed to make the “-122” promoter fragment contains a cluster of GC-rich DNA, which serves as binding sites for members of the Specificity Protein/Krüppel-like Factor (SP/KLF) transcription factor superfamily. Luciferase assays revealed that loss of this GC-rich 82-bp region results in reduced basal E2F1 transcription (**Figure 7B**), as noted previously by the Nevins lab [8]. Depending on the experiment, loss of this same region sometimes reduces R5020-mediated activation of E2F1 transcription. This prompted us to question whether a member of the Sp/KLF family could be involved in basal and/or R5020-mediated induction of E2F1 expression.

To investigate this issue, we began by pre-treating T47D cells with Mithramycin A, an antibiotic that binds to GC-rich DNA and blocks recruitment of Sp/KLF to these regions. We found that pre-treatment with Mithramycin A partially inhibits R5020-mediated induction of E2F1 (**Figure 8**). Mithramycin A does not inhibit induction of all PR target genes; in fact, some target genes are not affected at all, while others are induced. Next, we used real-time qPCR analysis to investigate whether PR regulates expression of any members of the SP/KLF transcription factor superfamily. Indeed, we found that R5020 induces several Sp/KLF family members - including Sp1, KLF4 and KLF9 - with KLF15 being the most robustly induced among those that we examined (**Figure 9**).

Next, we performed a knockdown experiment using Invitrogen's Stealth RNAi technology. Preliminary results show that siRNA knockdown of KLF15 reduces R5020-mediated induction of E2F1 expression (**Figure 10**). Based on previously characterized KLF binding sites, we constructed a positional weight matrix (PWM) to represent the DNA sequence motif that KLF15 recognizes. Using this matrix, we were able to identify several putative KLF15 binding sites within the 82-bp GC-rich region of the E2F1 promoter that is missing in the -122 luciferase construct (**Figure 11**). In future ChIP studies, we plan to determine whether PR-induced KLF15 is directly recruited to this region of the E2F1 promoter.

As we noted previously, Mithramycin A affects R5020-mediated induction of many downstream PR target genes, although a few target genes were not affected. Additionally, knockdown of KLF15 inhibits R5020 induction of several PR target genes. Therefore, a potential avenue of future exploration would be to determine whether the cooperation between PR and KLF15 and/or other SP/KLF family members in the regulation of E2F1 transcription represents a more global model of PR function. This could lead to a new mechanistic paradigm, whereby PR acts through multiple pathways, both direct and indirect, to achieve regulation of downstream target genes in T47D breast cancer cells (**Figure 12**).

Finally, we would like to mention a significant discovery that we made relating to the dose of progestin used in our experiments. The standard concentration of R5020 used in the field is 10 nM (or 10-8 M) R5020; at this dose of R5020, progesterone receptors within each cell are saturated with ligand, and are able to induce maximal activation of classical downstream target genes such as S100P (**Figure 13**). Therefore, the initial microarray study was done using T47D cells treated with 10 nM R5020, and all of the experiments conducted during the 2006-2007 reporting period were also done at that concentration of hormone.

At the beginning of the present reporting period, we decided to do a dose response experiment to make sure that we were treating cells with the optimal amount of progestin needed to achieve maximal E2F1 induction. To our great surprise, we saw a biphasic dose response curve, with lower concentrations of R5020 (100 pM, or 10-10 M) inducing the most robust E2F1 expression at both the mRNA (**Figure 13**) and protein (**Figure 14**) levels. After this discovery, we used 100 pM R5020 for all subsequent experiments, and repeated many of the key experiments done in the previous reporting period using the lower dose of R5020. In addition, we checked to see whether natural progesterone also regulates E2F1 expression in this manner. Preliminary studies show that E2F1 is induced in a similar biphasic fashion after treatment with increasing doses of progesterone (**Figure 15**).

We intend to further research this phenomenon, since it could have important clinical implications. Progestins are often used in combination with estrogens in hormone replacement therapy (HRT) for healthy menopausal women, due to the ability of progestin to block the proliferative and tumorigenic effects of estrogens in the uterus [9]. Unfortunately, the Women's Health Initiative (WHI) trial found that women receiving both hormones had an increased risk of invasive breast cancer compared to women receiving estrogen alone [4]. Therefore, the current

paradigm in hormone replacement therapy – and in contraceptives – has been to use as low a dose of progestins as possible. However, our data indicates that low dose progestins could be functioning to induce higher levels of E2F1 and other key regulators of cell cycle, which could have growth-stimulatory effects on breast cancer cells.

The **final task** for this project was to evaluate the E2F-PR axis as a target for therapeutic intervention. Since the relative induction of known PR target genes such as S100P by candidate selective PR modulators (SPRMs) does not always correlate with their effect on breast cancer cell proliferation, we plan to assess whether PR-mediated induction of E2F activity can be used as a more accurate marker to predict whether candidate SPRMs will evoke a proliferative response in breast cancer cells. Given that E2F activity is necessary for cell cycle progression from G1 to S phase, we expect to find that SPRMs which induce breast cancer cell proliferation also positively regulate E2F activity. If this proves correct, then E2F activity could potentially be used as a screen to identify novel SPRMs that inhibit the proliferation of hormone-dependent cancers. Additionally, we intend to determine whether there is a difference in subsequent proliferation of T47D breast cancer cells after treatment with high dose progestins versus low dose progestins.

Key Research Accomplishments

- Agonist-bound PR-B is recruited to the E2F1 promoter, where it can function in a direct and classical manner to induce transcription of E2F1.
- Treatment with R5020 stimulates increased recruitment of E2F1 to its own promoter in a positive feedback loop.
- Ligand-bound PR-B induces expression of KLF15, a member of the Sp/KLF superfamily of transcription factors, which can the bind to GC-rich DNA within the E2F1 promoter and further amplify transcriptional activation of E2F1.
- In T47D breast cancer cells, lower concentrations of the synthetic progestin R5020 are better able to mediate induction of E2F1 than higher concentrations of R5020.

Reportable Outcomes

Progesterone Receptor-Mediated Regulation of Cell Cycle in Breast Cancer. Ogden HE and DP McDonnell. Poster presented at Duke University Medical Center 16th Annual Biological Sciences Graduate Student Symposium, Durham, NC (November 9, 2007).

Progesterone Receptor-Mediated Regulation of Cell Cycle in Breast Cancer. Ogden HE and DP McDonnell. Poster presented at Era of Hope Department of Defense Breast Cancer Research Program Meeting, Baltimore, MD (June 25-28, 2008).

Regulation of E2F1 by Progestins. Wade HE and DP McDonnell. Poster presented at Duke University Medical Center Department of Pharmacology and Cancer Biology Annual Retreat, Wrightsville Beach, NC (September 26-28, 2008).

Conclusion

Our studies show that agonist-bound PR-B can stimulate downstream E2F signaling in T47D breast cancer cells by functioning in both a direct and indirect manner to induce transcription of E2F1 (**Figure 12**). In the future, a potential avenue of further exploration would be to determine whether the cooperation between PR and KLF15 and/or other SP/KLF family members in the regulation of E2F1 transcription represents a more global model of PR function. If a substantial subset of downstream target genes is regulated by PR in a similar

multimodal fashion, then each novel pathway should be evaluated as a potential target for therapeutic intervention.

In addition, we have discovered that low-dose progestins are better able to activate transcription of downstream PR target genes related to cell cycle regulation than high-dose progestins. This finding has significant clinical implications, given that low-dose progestins are used widely in combination HRT and contraceptives, and future work will be done to probe the mechanisms behind this phenomenon.

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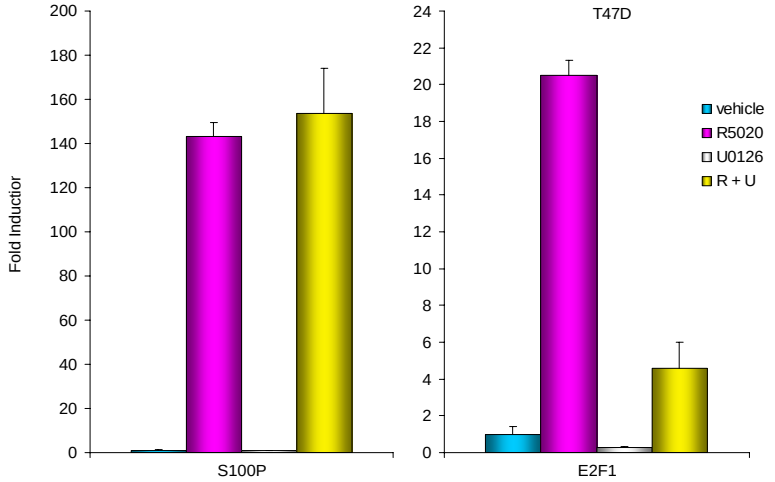
Appendices

None.

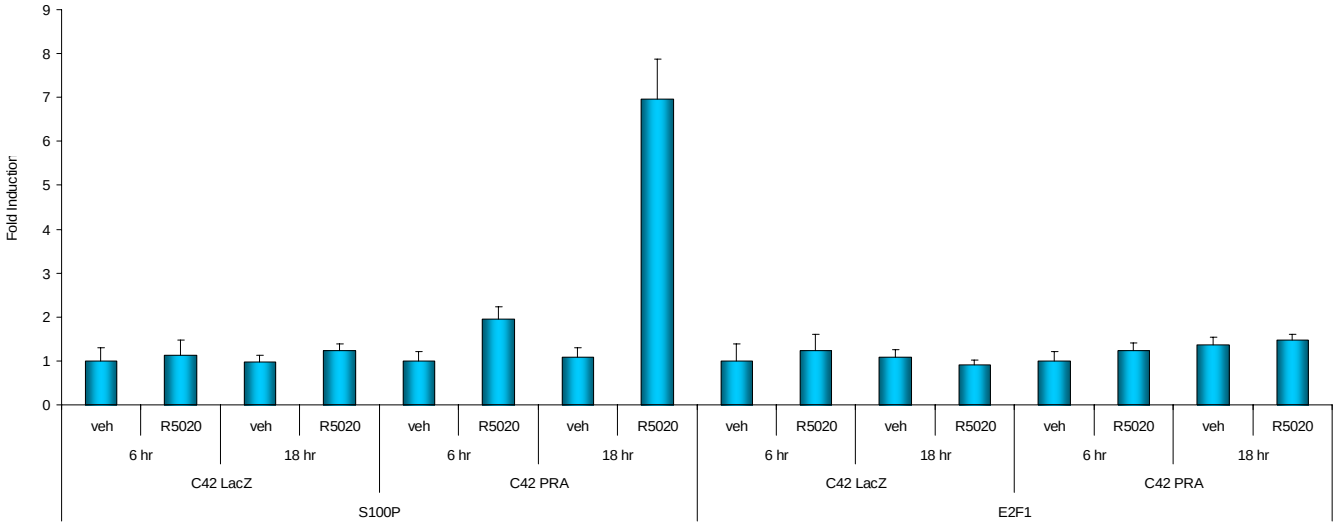
Supporting Data

Figure 1. R5020 induces expression of endogenous E2F1 mRNA in T47D breast cancer cells.



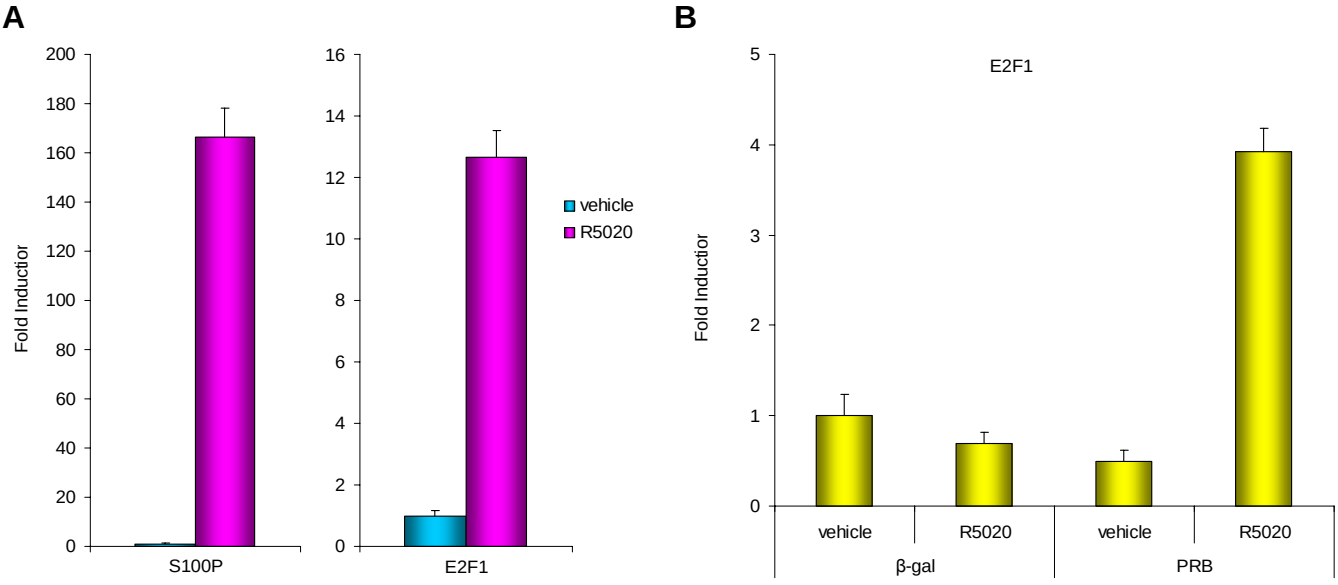
Synchronized T47D cells treated with vehicle or 100 pM R5020 +/- 10 μM U0126 were harvested after 18 hours. Real-time qPCR analysis shows that R5020-mediated induction of E2F1 mRNA levels in T47D cells is repressed by U0126.

Figure 2. R5020 does not induce E2F1 mRNA in PR- T47DC42 cells expressing LacZ or PRA alone.



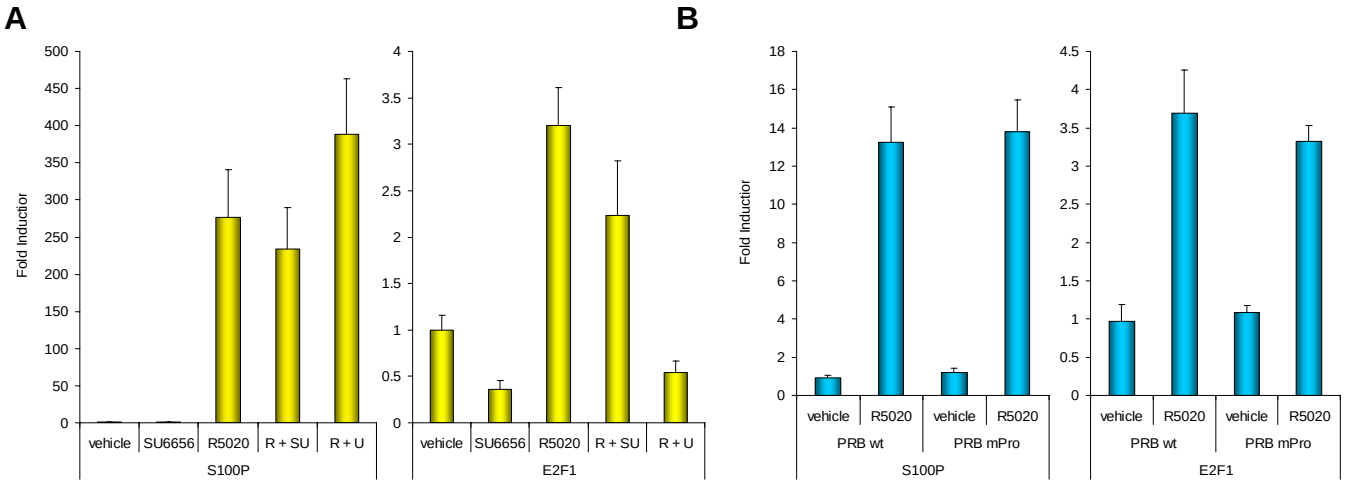
Synchronized T47DC42 cells were treated with vehicle or 10 nM R5020 for 6 or 18 hours, and harvested. Real-time qPCR analysis shows that R5020 is unable to mediate induction of E2F1 mRNA in T47DC42 cells lacking PRB.

Figure 3. R5020 induces E2F1 mRNA in BT483 breast cancer cells and in HMECs infected with a PR-B adenovirus.



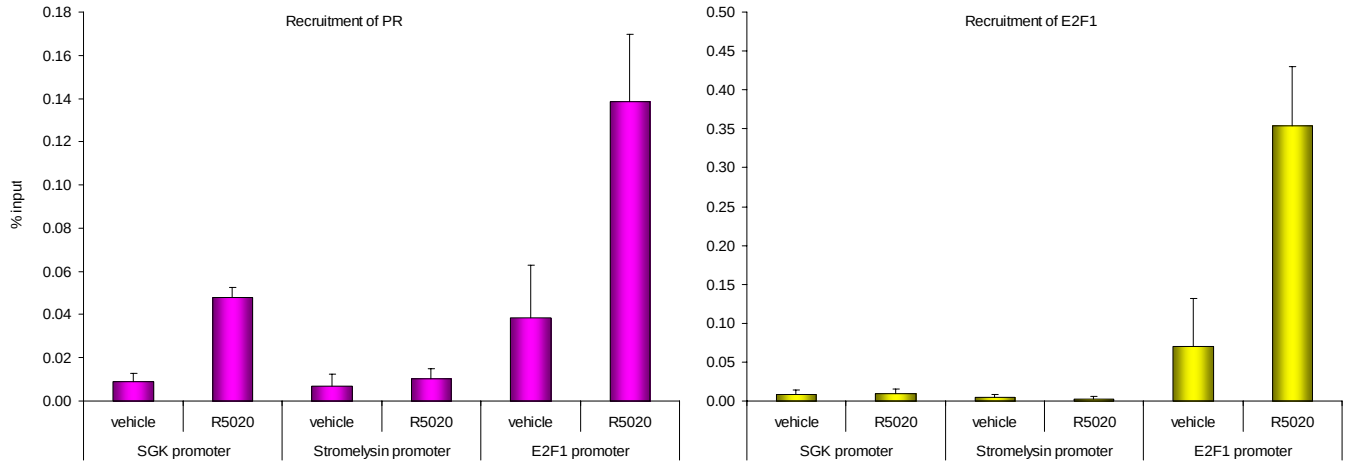
(A) BT483 breast cancer cells and (B) HMECs infected with a control β-gal or PR-B adenovirus were treated with vehicle or 10 nM R5020 for 18 hours. Real-time qPCR analysis shows that R5020 mediates induction of E2F1 mRNA levels in both cell lines.

Figure 4. PR signaling through Src family kinases is not required for R5020-mediated induction of E2F1 expression.



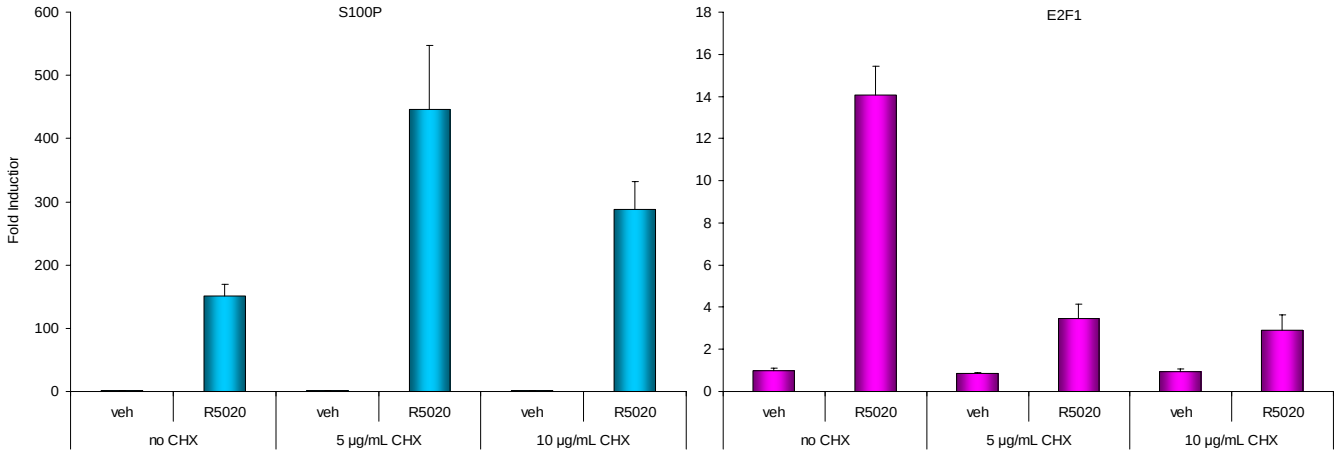
(A) T47D cells were treated with vehicle or 10 nM R5020 +/- 1 μM SU6656 or 10 μM U0126 for 18 hours and harvested. Real-time qPCR analysis shows that R5020-mediated induction of E2F1 mRNA levels in T47D cells is not abrogated by SU6656. (B) T47DC42 cells were treated with vehicle or 10 nM R5020 for 18 hours and harvested. Real-time qPCR analysis shows that R5020 induces similar E2F1 mRNA levels in cells expressing wild-type PR-B vs. the mutant PRBmPro version.

Figure 5. PR and E2F1 are recruited to the E2F1 promoter in the presence of R5020.



Synchronized T47D cells were treated with vehicle or 100 pM R5020 for 60 minutes and subjected to ChIP analysis. Cross-linked chromatin fragments were immunoprecipitated with a PR- or E2F1-specific antibody and analyzed using real-time qPCR. Treatment with R5020 significantly increases recruitment of PR and E2F1 to the E2F1 promoter.

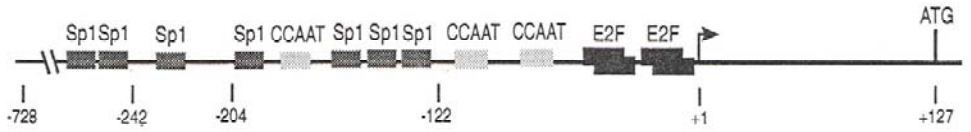
Figure 6. Cycloheximide inhibits R5020-mediated induction of E2F1 transcription.



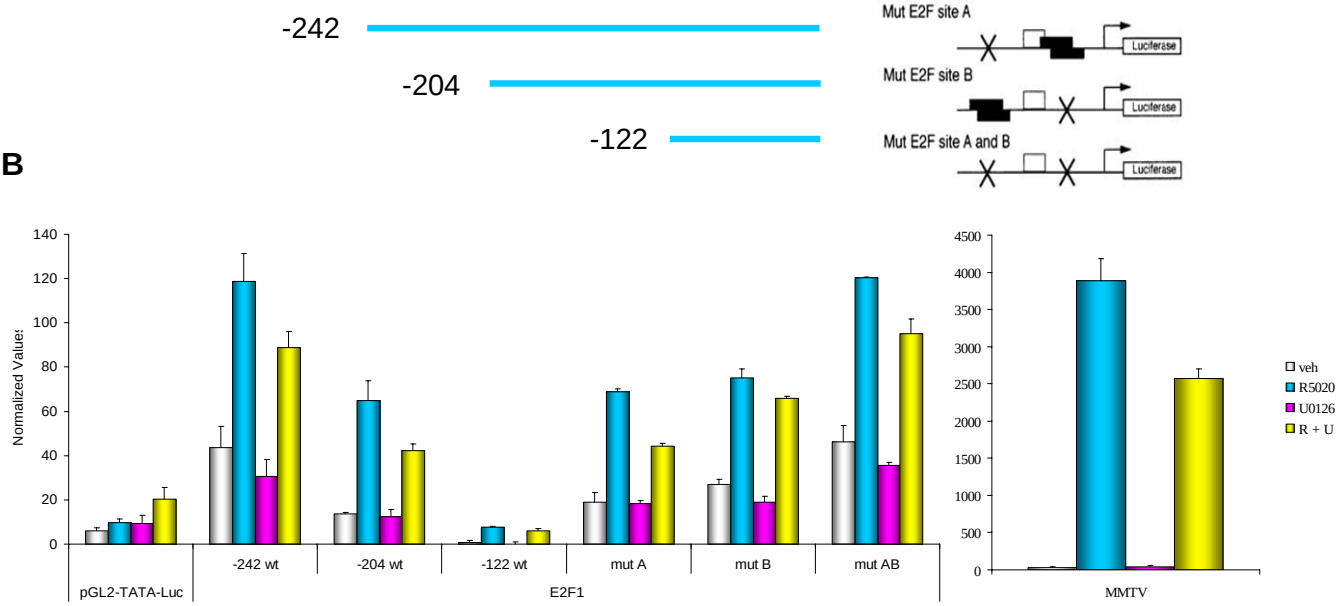
Synchronized T47D cells were treated with vehicle or 100 pM R5020 +/- 5 or 10 µg/mL cycloheximide for 18 hours and harvested. Real-time qPCR analysis shows that R5020-mediated induction of E2F1 mRNA levels in T47D cells is inhibited by cycloheximide, which blocks *de novo* protein synthesis.

Figure 7. A closer look at R5020 activation of different E2F1 promoter fragments.

A

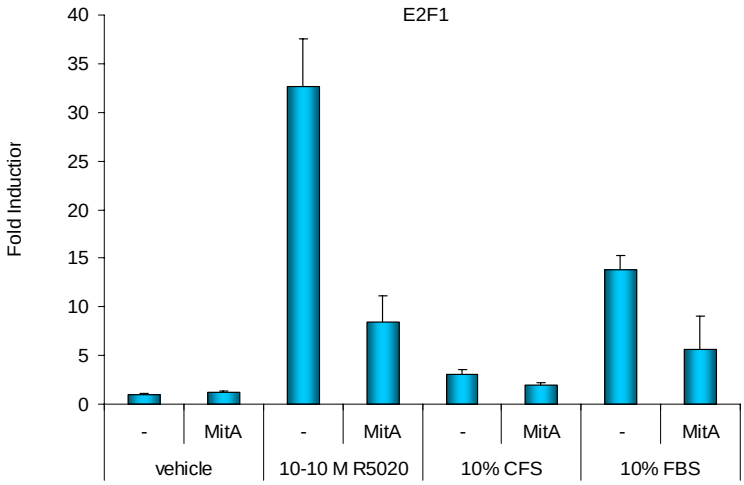


B



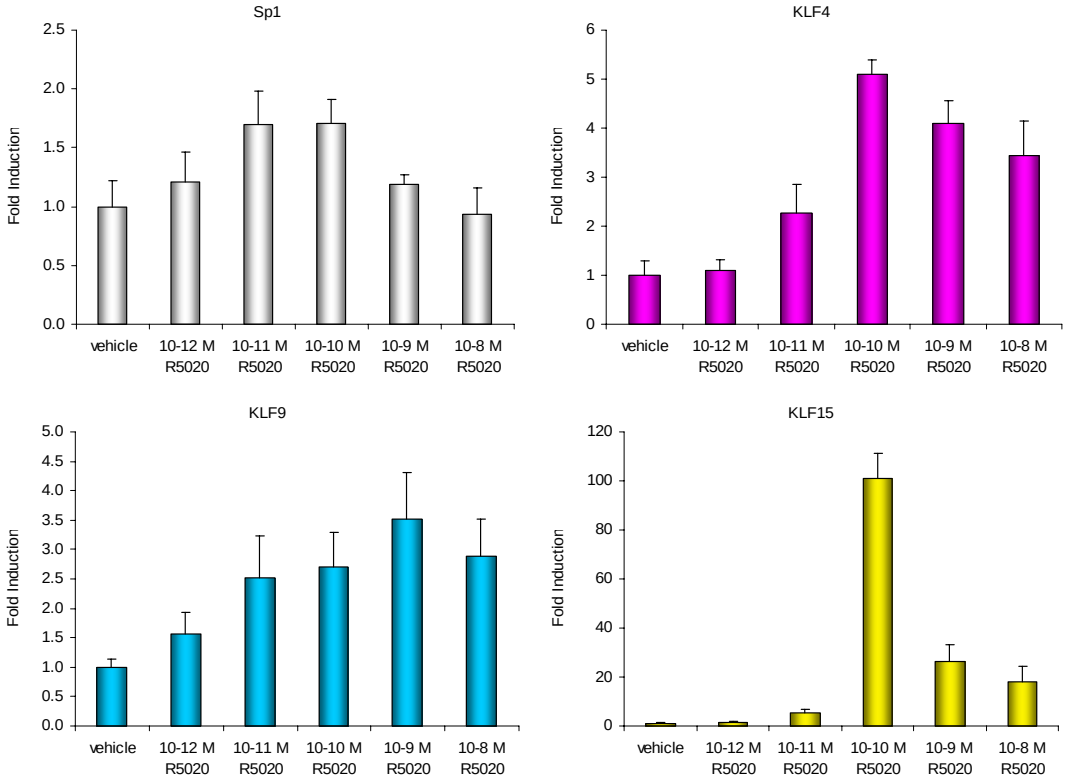
(A) Layout of E2F1 promoter fragments/mutants [8]. (B) T47D cells were transiently transfected with various hE2F1-luc promoter fragments, (-) control pGL2-TATA-Luc (empty vector), or (+) control MMTV reporter constructs; treated with vehicle or 10 nM R5020 +/- 10 µM U0126 for 24 hours; and assayed for luciferase activity. Loss of the GC-rich 82-bp region in the -122 fragment results in reduced basal E2F1 transcription.

Figure 8. Mithramycin A inhibits R5020-mediated induction of E2F1 expression.



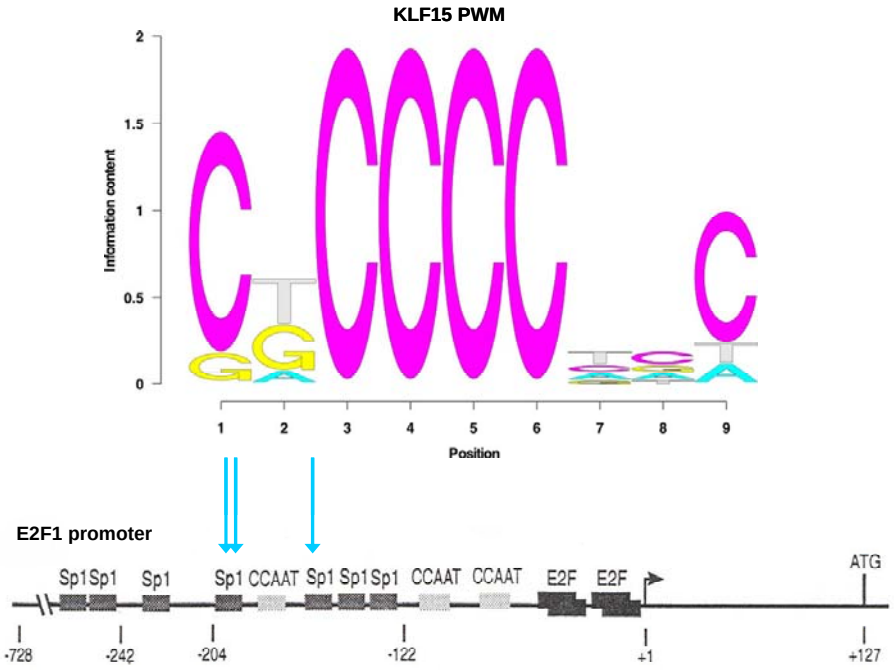
Synchronized T47D cells were pre-treated for 0.5 hours with 200 nM Mithramycin A, then treated with vehicle, 100 pM R5020, 10% charcoal-stripped serum, or 10% full serum for 18 hours and harvested. Real-time qPCR analysis shows that pre-treatment with Mithramycin A inhibits R5020-mediated induction of E2F1 expression.

Figure 9. R5020 induces expression of several Sp/KLF family members.



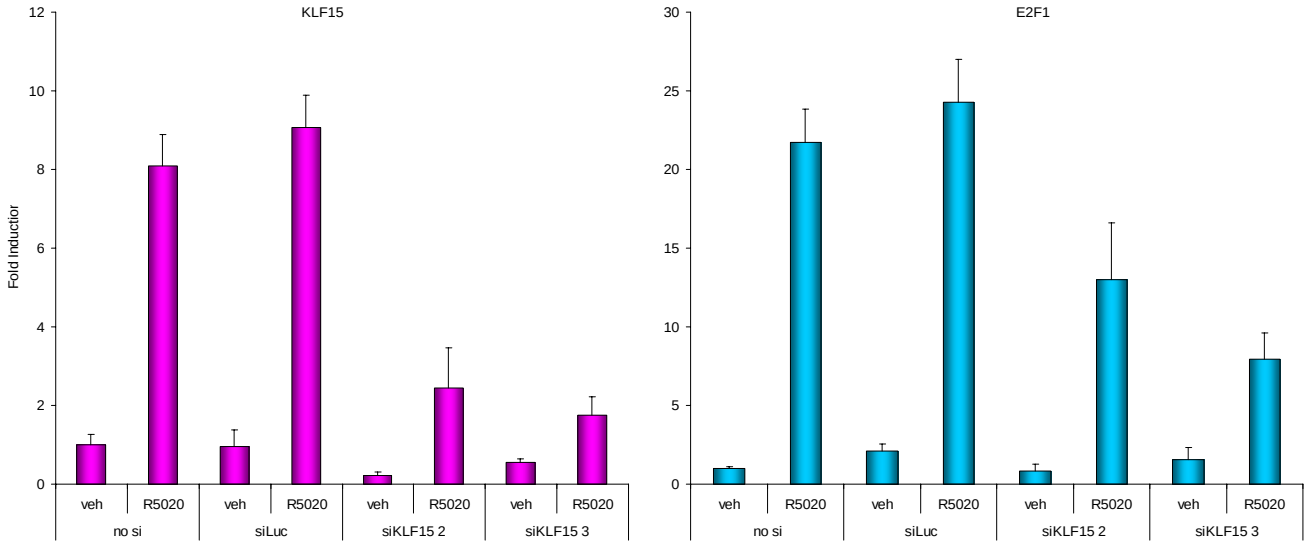
Synchronized T47D cells were treated with vehicle or varying doses of R5020 for 18 hours, and harvested. Real-time qPCR analysis shows that R5020 induces expression of Sp1, KLF4, KLF9 and KLF15.

Figure 10. Identification of putative KLF15 binding sites within the E2F1 promoter.



A positional weight matrix (PWM) was constructed based on previously characterized KLF binding sites and used to identify putative KLF15 binding sites within a GC-rich region of the E2F1 promoter.

Figure 11. Stealth siRNA knockdown of KLF15 inhibits PR regulation of E2F1 transcription.



T47D cells were transfected with Stealth siRNA for 48 hours (siLuc = (-) control), synchronized by serum-starvation for 24 hours, then treated with vehicle or 100 pM R5020 for 18 hours and harvested. Real-time qPCR analysis shows that induction of E2F1 mRNA by R5020 is inhibited in cells with KLF15 knockdown.

Figure 12. Multimodal regulation of E2F1 by progestins.

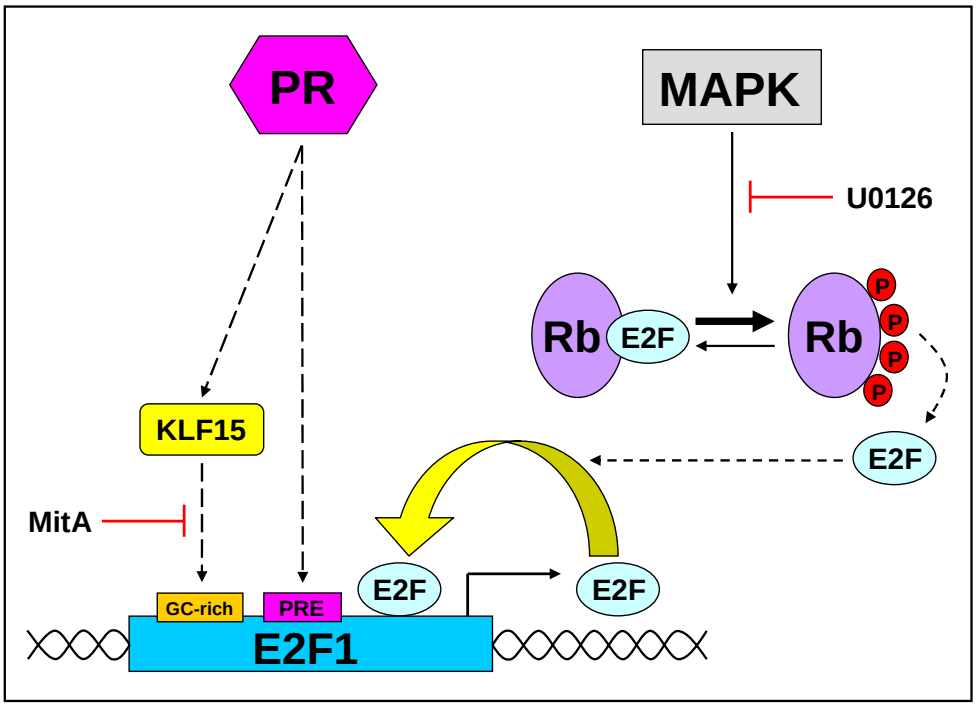
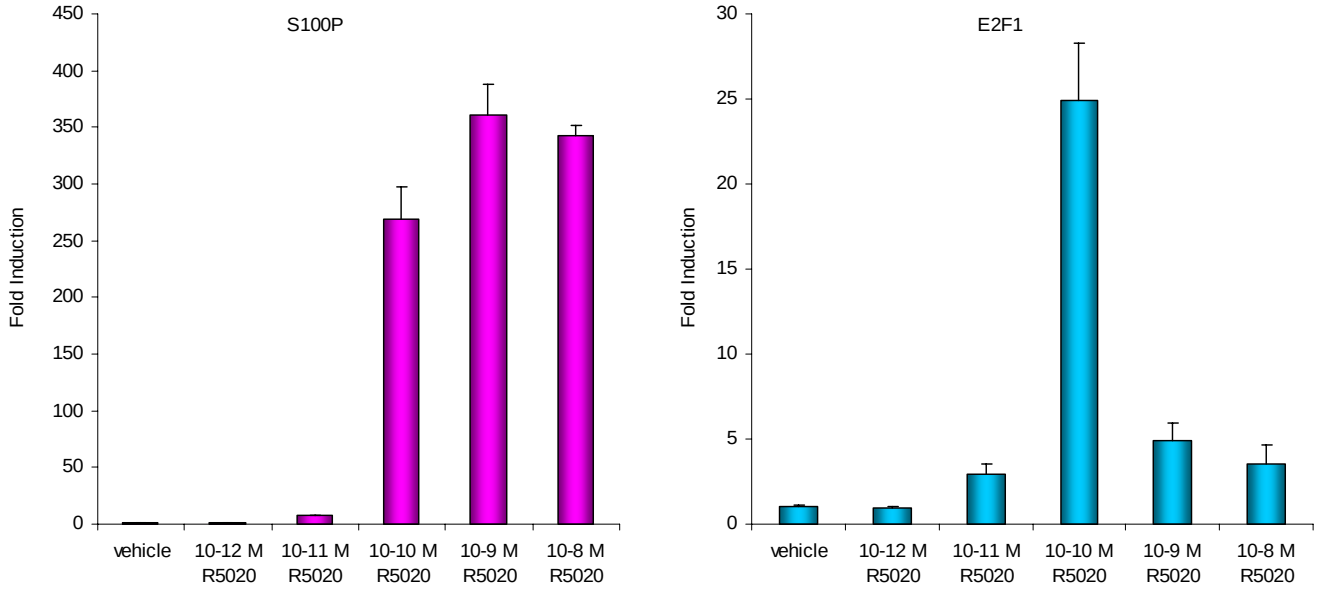
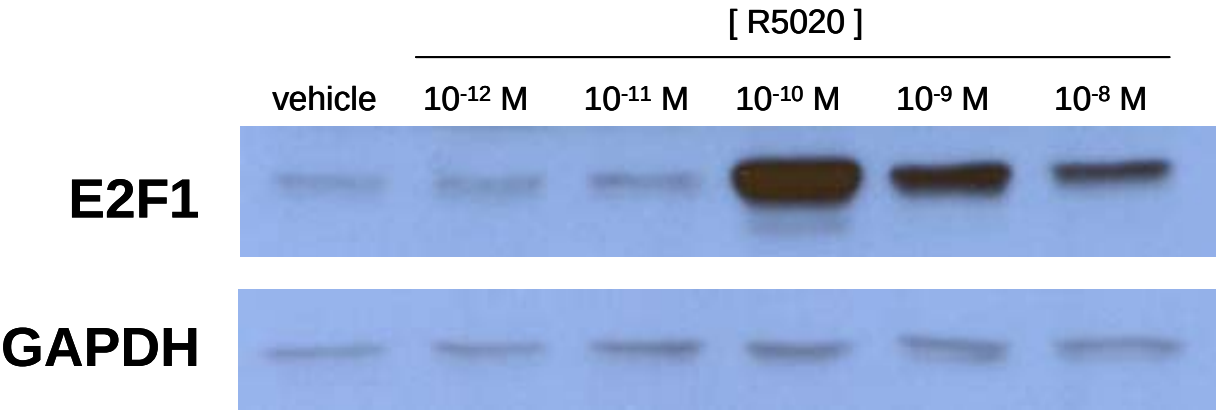


Figure 13. R5020 dose response curves for classical PR target gene S100P vs. E2F1.



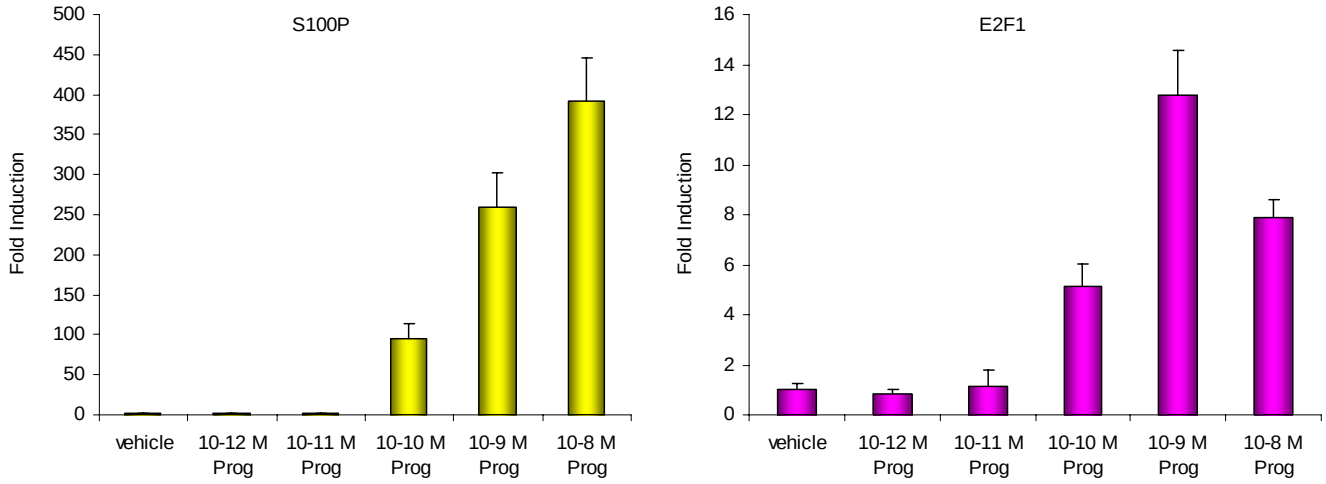
Synchronized T47D cells were treated with vehicle or varying doses of R5020 for 18 hours and harvested. Real-time qPCR analysis shows that induction of S100P by R5020 follows a classic dose response curve, with maximal activation at the highest concentrations of R5020. However, R5020-mediated induction of E2F1 displays a biphasic dose response curve; lower concentrations of R5020 (100 pM, or 10^{-10} M) induce the most robust E2F1 expression, while higher concentrations of R5020 (10 nM, or 10^{-8} M) are not able to achieve maximal induction of E2F1 expression.

Figure 14. R5020 induces expression of endogenous E2F1 protein in T47D cells.



Synchronized T47D cells were treated with vehicle or varying doses of R5020 for 18 hours, and harvested. Western blot analysis shows that 100 pM R5020 is able to induce the most robust levels of E2F1 protein, and confirms the biphasic dose response curve seen at the RNA level in Figure 13.

Figure 15. Progesterone induces expression of endogenous E2F1 mRNA in T47D breast cancer cells.



Synchronized T47D cells were treated with vehicle or varying doses of progesterone for 18 hours, and harvested. Real-time qPCR analysis shows that induction of S100P by progesterone follows a classic dose response curve, with maximal activation at the highest concentrations of R5020. However, progesterone-mediated induction of E2F1 displays a biphasic dose response curve; lower concentrations of progesterone (1 nM, or 10^{-9} M) induce the most robust E2F1 expression, while higher concentrations of progesterone (10 nM, or 10^{-8} M) are not able to achieve maximal induction of E2F1 expression.